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I, LEANNE MYNOTT, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PP 6165 for a patent by THE UNIVERSITY OF QUEENSLAND filed on 25 September 1998.

WITNESS my hand this
Tenth day of November 1999

L. Mynott

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SUPPORT AND SALES

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Patents Act 1990

PROVISIONAL SPECIFICATION

Applicant(s): THE UNIVERSITY OF QUEENSLAND

Invention Title: AUXILIARY FOR AMIDE BOND FORMATION

The invention is described in the following statement:

AUXILIARY FOR AMIDE BOND FORMATION

This invention relates to novel auxiliaries for the formation of amide bonds, and to the use of these auxiliaries in a variety of synthetic applications. In particular, the auxiliaries of the invention are useful in the synthesis of peptides and peptidomimetic compounds, and in particular for the synthesis of "small cyclic peptides", so-called "difficult" peptide sequences, and large peptides with a native peptide backbone. The auxiliaries of the invention are also useful in the synthesis of peptides or of C-terminal modified peptides, and in on-resin cyclisation of organic molecules, ligating chemistry, backbone substitution and as backbone linkers. In a particularly preferred embodiment, the invention provides auxiliaries which can be removed by photolysis.

BACKGROUND OF THE INVENTION

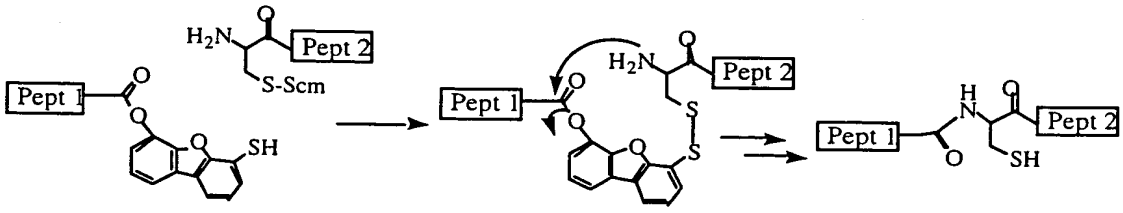
Amide bond formation is one of the most studied reactions in chemistry and biology. It allows peptide and protein synthesis, and enables the synthesis of peptide-like molecules, known as peptidomimetic compounds, which are widely used in drug design and discovery programs. A plethora of reagents and reaction conditions have been developed over the years that facilitate amide bond formation by activating a carboxylic acid and mixing it with a primary or secondary amine. In a number of cases however the acylation reaction may not go to completion or may not proceed at all. Despite the progress and extensive research efforts in this field, so-called "difficult" amide bonds still exist that prevent access to a large number of compounds of great interest to the research community. In particular these include small cyclic peptides, large peptides and proteins and difficult peptide sequences. In these cases, attempts to force the acylation by heating or by increasing the activity of the activated ester result in undesirable side reactions, such as

racemisation, or dimerisation. In these cases a different approach is required to facilitate the amide bond formation. In the past 10 years a number of auxiliary strategies have been developed that make use of an intramolecular acyl transfer to overcome some of these problems. These strategies and their targets are outlined in more detail below.

1. Native ligation chemistry

The general idea of chemical ligation is to synthesise large proteins in high purity. The process capitalises on the ability to generate highly homogeneous linear peptides of up to 50 residues long by using optimised solid phase peptide synthesis. These peptide segments are then linked or ligated in solution, using mutually reactive entities at the end of each segment. The major limitation to the existing ligation strategies is that they only work for a very limited number of ligation sites.

Several examples have been published where mutually reactive groups generate an amide isostere. In these first examples the ligation chemistry produced a modification in the peptide backbone of the product. In 1986, Kemp *et al* (1986) proposed a thiol-capture strategy, which is illustrated in Scheme 1.



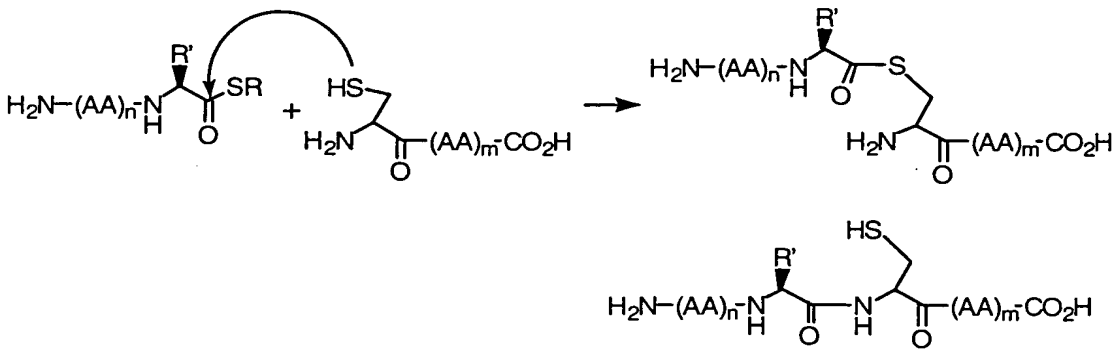
Scheme 1

The thiol-capture strategy

Here two peptide segments are ligated using a mercaptobenzofuran substituent at the C-terminus of the first segment. The second segment, carrying a cysteine

residue at the N-terminus, reacts to form a disulfide link. Following an *O*-to-*N*-acyl shift the disulfide link is cleaved, generating a "native" amide bond. This auxiliary strategy, although revolutionary in its own right, lacks versatility and has only been used successfully in a very limited number of cases (Fotouhi *et al*, 1989) due to the inherent difficulties in synthesising the selectively-protected peptide segments. It differs from our invention in the strategic approach and the design of the auxiliary. The same group has studied a number of parameters that influence the rate of the intramolecular acyl transfer, mostly focussing on the shape of the auxiliary (Kemp *et al*, 1981). This work is very different from our invention, and in no way suggested anything that is described in this invention.

In 1994 Dawson *et al* (1994) introduced the concept of native ligation, which allows the generation of proteins with a native or unmodified backbone from fully unprotected building blocks. This approach, outlined in Scheme 2, uses chemistry first described by Wieland for reacting amino acids.



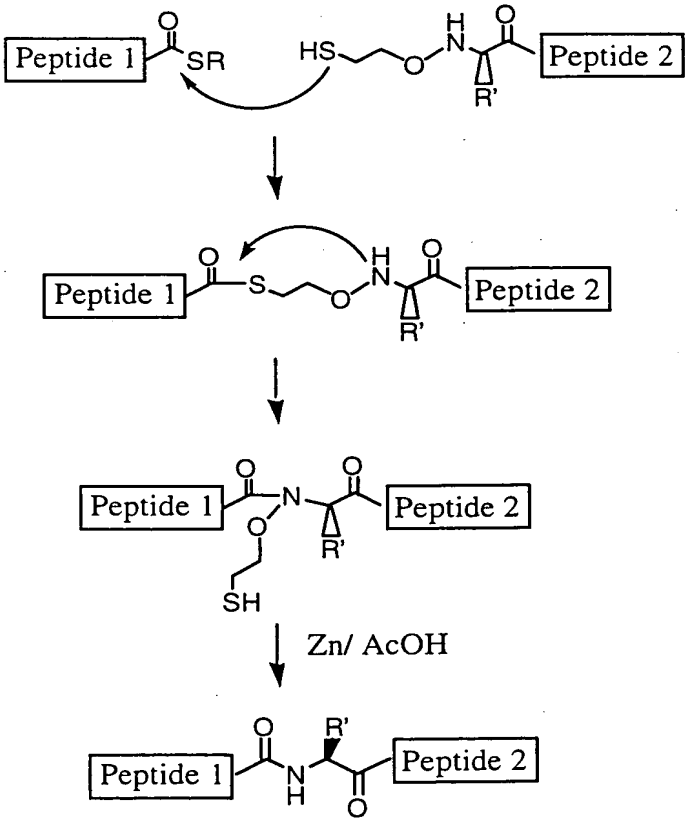
Scheme 2

"Native ligation"

In a first step an unprotected peptide-α-thioester selectively reacts with the thiol functionality on the N-terminal cysteine side chain of a second

unprotected peptide. The initially-formed thioester undergoes a spontaneous acyl transfer in the aqueous buffer from a sulfur to a nitrogen atom, thereby generating a standard peptide bond. Several examples illustrate the significance of this work in enabling the synthesis of a large proteins in high purity (Hackeng *et al*, 1997). One of the limitations of this native ligation strategy is that it relies on the presence of a cysteine residue somewhere in the middle of the target peptide sequence.

In an extension of this work, Canne *et al* (1996) reported a native ligation strategy that uses an auxiliary at the N-terminus of one of the peptide segments. The strategy avoids the necessity for an N-terminal cysteine residue, and expands the range of sites amenable to native chemical ligation to X-Gly and Gly-X. The strategy is outlined in Scheme 3.



Scheme 3

Extension of native ligation

A peptide-α-thioester reacts with an N^{α}(oxyethanethiol) peptide to produce the ligated product. The thioester-linked intermediate rearranges via an acyl transfer to an amide-linked product. The N-O bond at the tertiary amide can be readily cleaved using zinc dust in acidic aqueous solution, thereby releasing the oxyethanethiol auxiliary and producing the native backbone structure in the ligated product. The scope and limitation of this auxiliary approach was examined by selecting a range of different ligation sites.

It was found that the S-to-N acyl transfer only proceeded well for Gly-Gly ligation sites, but was more difficult when steric hindrance around the ligation site was increased. For example, in the case of a Gly-Ala ligation site the rearrangement, was incomplete after 10 hours in the pH 7.5 buffer. Lowering the pH to 4.5 accelerated the rearrangement, which was complete after 10 hours. In the case of a Phe-Gly ligation site rearrangement was almost complete after 2 days at 37°C. For the more hindered Phe-Ala ligation site no *S*-to-*N* acyl transfer step was observed, even after lowering the pH or leaving the sample at 37°C for 24 hours. It was thus concluded that only Gly-X or X-Gly ligation sites will produce the target product. The limitation lies in the acyl-transfer step, which does not proceed for hindered ligation sites. The strategy differs from our invention in the way the auxiliary is introduced and removed, and in the design of the auxiliary.

Native ligations have also been performed using resin-bound peptides. One such strategy (Camarero *et al*, 1998) involves the assembly of a first peptide segment linked via a C-terminal thioester to the solid support, then adding the second segment containing a cysteine residue at the N-terminus, and performing the native ligation steps as for solution phase ligation. This has the advantage that handling of the intermediates is

significantly reduced. Furthermore, several ligations can be performed in series using the same chemical approach. The limitations for the solid phase approaches are the same as for the solution phase chemistry, ie native ligation can only be performed at X-Cys, Gly-X or X-Gly sites.

2. Small cyclic peptides

Proteins and peptides are the primary means of initiating biological processes by interacting with macromolecular receptors. The crucial information determining the specific activity is often contained in relatively small sequences at the surface, and is determined by the three-dimensional conformation in which that sequence positions its side chains when interacting with the receptor. In the linear form, bioactive peptides can assume millions of different conformations, only very few of which are able to bind to the target receptor. In order to assess the important structural and dynamic properties that are critical to the biological potency and selectivity, conformational constraints are introduced, typically through cyclisation. Such cyclic molecules exist in more defined conformations, and are therefore very appealing from the point of view of pharmaceutical lead discovery. If activity is maintained or enhanced in these cyclic peptides, structural information is obtained, for example by NMR, X-ray or molecular modelling, and used to guide the development of therapeutic drugs. In addition, cyclisation generally promotes an increase of metabolic stability and bioavailability of peptides.

As the side chains are considered the main mediators for receptor interaction, cyclisation is preferably accomplished between the C- and N-termini. Whereas the synthesis of linear peptides generally proceeds well, head-to-tail cyclisation is often troublesome. This is particularly so for small peptides, *ie.* those less than seven residues long. All-L cyclic tetrapeptides for instance are not very accessible (Schmidt and Langner,

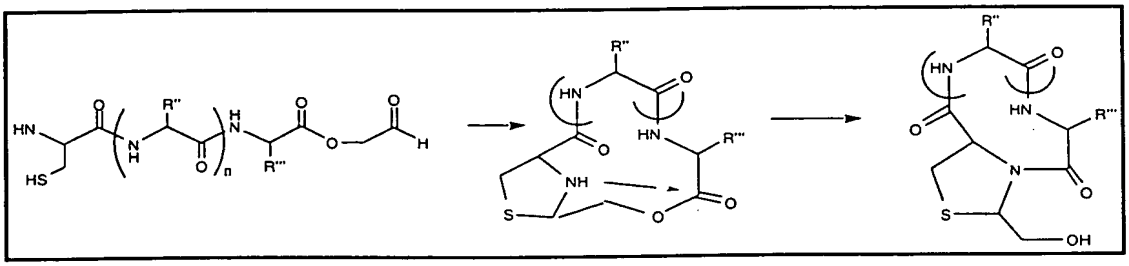
1997). The primary reason for ineffective cyclisation originates from what are called "difficult sequences". In cyclisation terms this refers to a sequence-related inefficiency in "bringing the ends together" for head-to-tail cyclisation. Peptide bonds have strong π-character, and preferentially adopt a trans conformation. Linear precursors are therefore generally extended in conformation with terminal carboxylic acid and amine functional groups in remote positions, and are thus unfavourable for cyclisation. The problem is most prominent in the synthesis of small cyclic peptides, where activation of the C-terminus often results in the formation of linear and cyclic dimers or oligomers with low or no yield of target cyclic monomer.

There have been very few studies that address the "difficult" cyclisation issue. Cavalier-Frontin *et al* (1993) reported on the use of reversible chemical modifications of the peptide backbone to enhance cis-amide conformations. In the synthesis of cyclo-[Phe-Phe-Phe-Phe], each amide N was substituted with a BOC protecting group. The cyclisation yield increased from 1% to 27%. Similarly, the use of the N-(2-hydroxy-4-methoxybenzyl) (Hmb) group as a reversible N-backbone amide substituent has resulted in increases in yield of cyclic peptides (Ehrlich *et al*, 1996). It must be emphasised that here the "auxiliary" is placed on the backbone amide, and not on the N-terminal amine that reacts to form the "difficult" amide bond.

In the past two to three years a few studies where ligation chemistry was used in an intramolecular fashion have been reported. In these examples an initially larger ring is formed, and ring contraction accomplished through an intramolecular O-to-N or S-to-N acyl transfer.

In a first method reported by Botti *et al* (1996), linear unprotected peptides carrying a cysteine residue at the N-terminus and an aldehyde at the C-terminus, were cyclised to generate a thiazolidine containing cyclic

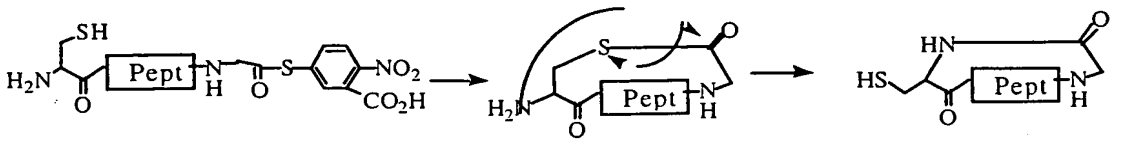
peptide, as shown in Scheme 4. Initially a larger cycle is formed, in which the C- and N- termini are prepositioned for O-to-N acyl transfer and ring contraction to a smaller cycle. The disadvantage of this method is that the cyclic product always contains a thiazolidine moiety in the cycle, with an additional chiral centre which results in the formation of two diastereomers, and requires a cysteine residue at the N-terminus of the linear precursor. The method does not allow the generation of unmodified cyclic peptides, and is not a versatile procedure suitable for a combinatorial library approach.



Scheme 4

Ring contraction chemistry using a thiazolidine unit in the ring

Muir *et al* demonstrated that "native" ligation, using a cysteine residue at the N-terminus and a thioester at the C-terminus, can be applied in an intramolecular way to generate cyclic peptides (Camarero and Muir, 1997), as shown in Scheme 5.



Pept = YAVTGRGDSPAASS ≡ cyclo-[CYAVTGRGDSPAASSG]

Scheme 5

Intramolecular native ligation using cysteine

A 15-residue unprotected peptide containing a C-terminal thioacid was converted to the head-to-tail cyclic peptide by dissolving the activated C^{α}-thioester in a pH 7.5 buffer. Cyclisation was complete in 10 minutes. The initially formed cyclic thioester rearranges quickly to form the final peptide bond. The strategy is not generic, as it requires a cysteine residue at the N-terminus.

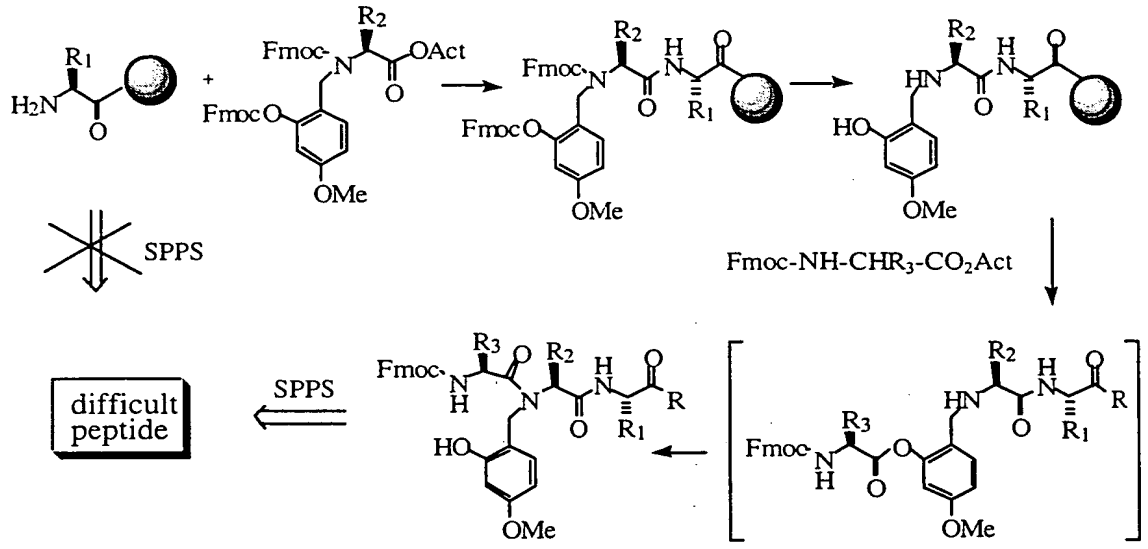
In a similar way Shao *et al* (1998) showed that N-(oxyethanethiol)-glycine at the N-terminus can be employed to achieve cyclisation by allowing the thiol functionality to react regioselectively with a thioester at the C-terminus. These strategies, as the authors point out, are limited by the types of residues involved at the C-and N-termini. Cyclisation is only possible between Gly-X, where X is a non β-substituted residue. The slow acyl transfer is again the limiting factor in this cyclisation strategy.

None of these methods provides a versatile synthetic route to enable synthesis of cyclic peptides with unmodified or native peptide backbone. The first two methods require the presence of cysteine at the N-terminus and the last lacks versatility, as the ring contraction only proceeds for non-hindered cases. We have found that the latter approach does not provide access to a number of known "difficult" cyclic peptides.

3. Backbone substitution

One of the major problems in solid phase peptide synthesis (SPPS) is the inefficient assembly of the so-called "difficult" sequences. Moreover, these sequence-related difficulties are impossible to predict *a priori*. The problems are believed to be mainly due to inter- and intra-chain aggregation during the assembly of the protected peptide on the solid support. This has led to the development of the backbone substitution strategy (Hyde *et al*, 1994) outlined below in Scheme 6. A 2-hydroxy-4-methoxybenzyl substituent (=Hmb) is introduced by using

N,O-bis-Fmoc-protected (Hmb)-amino acids. In general acylation of N-substituted amino acids other than glycine requires forcing conditions, due to the massive steric hindrance imposed by the N-substituent. In the case of the Hmb-substituted amino acids, acylation is substantially enhanced through an internal acyl transfer mechanism. Acylation initially occurs on the phenolic oxygen atom, enabled by the intramolecular presence of an amine base, and is followed by an acyl transfer from the oxygen to the nitrogen atom. Fmoc-solid phase synthesis then proceeds, with significantly improved yields for peptide sequences that are difficult to assemble using standard SPPS.



= solid support

Scheme 6

The use of Hmb-backbone protection during solid phase peptide synthesis

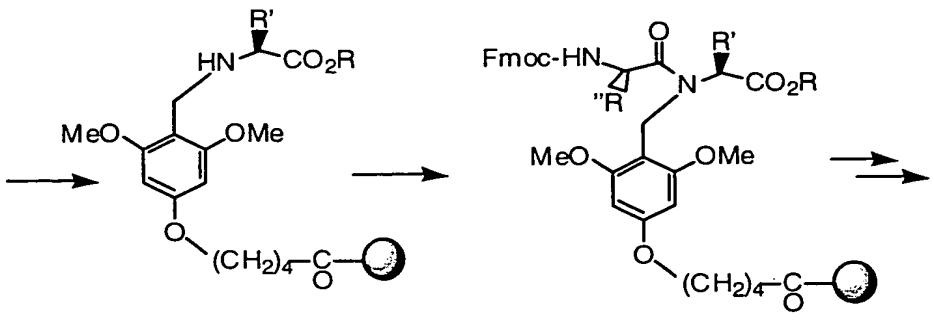
After assembly the peptide is deprotected and cleaved using TFA, with concurrent removal of the Hmb backbone substituent yielding target unprotected peptide in high yields and purity. This backbone protection can also

be employed to prevent aspartimide formation, and to improve solubility of protected peptides. In a recent report Hmb groups were introduced on resin-bound peptide via reductive amination, thereby avoiding the use of the more tedious N,O-bis-Fmoc (Hmb)amino acids (Nicolas *et al*, 1997).

There are two major limitations in the Hmb-backbone protection strategy. Firstly the internal O-to-N acyl transfer only proceeds well for non-hindered cases. When a β-branched amino acid has to be coupled to a N^{α}-Hmb residue other than glycine several hours of heating (80°C) is required for the rearrangement to proceed. Secondly, this group is only compatible with Fmoc chemistry, and not with the often-preferred BOC SPPS, due to its TFA lability.

The Hmb methodology has demonstrated that backbone substitution can alleviate sequence related assembly problems for Fmoc chemistry. However, for hindered cases it creates additional problems of its own. The methodology would benefit significantly from the development of a more acid stable auxiliary that would allow a faster intramolecular acyl transfer (that does not require heating) and improved assembly of difficult sequences using either Fmoc or Boc SPPS.

This Hmb-backbone substitution approach has led to the recent development of backbone amide linkers (BAL) (Jensen *et al*, 1998), as shown in Scheme 7.

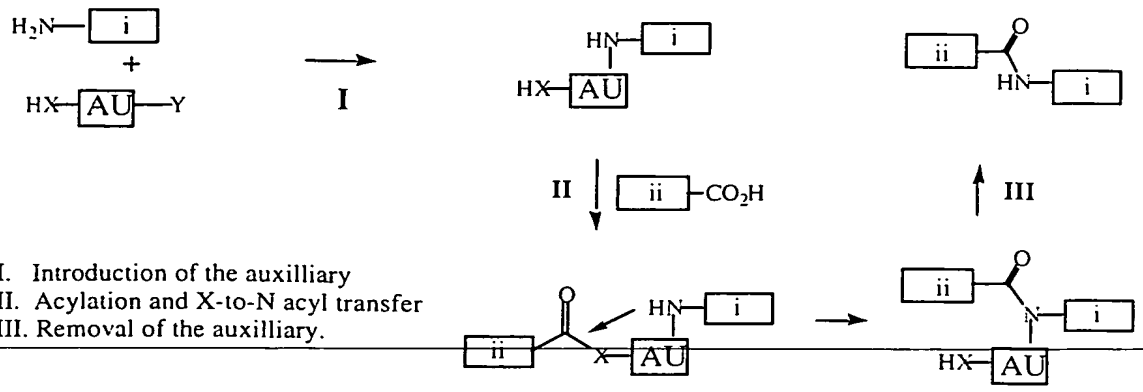


Scheme 7

The Backbone linker approach (BAL)

A tris-alkoxybenzyl unit is employed to link a peptide via the backbone amide nitrogen atom to a solid support. The link is cleaved by simple TFA treatment at the end of the synthesis. This linking strategy, in contrast to most other such strategies, does not make use of the C-terminal carboxylic acid, and can, at least in theory, be used on any amide bond. It is thus especially useful for synthesis of C-terminaly modified peptides or for the on-resin synthesis of head-to-tail cyclic peptides. As for the Hmb group, the first limitation lies in the difficulties of acylating the secondary amine to form the "linked" amide bond. A second problem is that standard Fmoc SPPS leads to almost complete diketopiperazine formation at the dipeptide stage. Special protection strategies need to be employed to avoid this problem.

The most valuable auxiliary strategies for peptide ligation, cyclisation or difficult peptide sequence assembly generate unmodified peptide backbones in the final product. There are three critical features in these auxiliary strategies: introduction, acylation and removal, as illustrated in Scheme 8. The prior art strategies have been successfully applied in a limited number of cases. However, applications of these strategies are severely limited by the difficulties encountered in the acyl transfer step and/or the final auxiliary removal. Often the acyl transfer is very slow, or does not proceed at all.



Scheme 8

Reaction steps in the auxiliary strategies

There are at least three requirements needed to make the auxiliary approach more versatile:

1. allow generic introduction of the auxiliary at the N atom,

2. allow more effective acylation of the nitrogen atom, and

3. allow removal of the auxiliary after acylation.

This combination of requirements severely limits the design of novel auxiliaries.

We have surprisingly found that a modification of the molecular fragment that links an oxygen or sulfur atom to the nitrogen atom has a strong accelerating effect on the acylation rate of the nitrogen atom, in contrast to prior art examples. In a particularly preferred embodiment, the modification further allows photolytic cleavage of the covalent bond between the acylated nitrogen atom and the remaining molecular fragment that connects the nitrogen atom with the oxygen or sulfur atom.

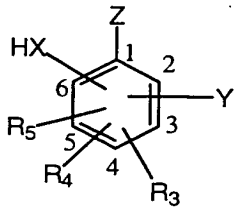
This approach is particularly useful in the field of peptide synthetic chemistry for applications such as formation of small cyclic peptides, formation of large peptides through native ligation of smaller peptide fragments, synthesis of "difficult" peptides, and backbone-linking to a solid support. The prior art methods are

often not effective, *ie.* they only work for a small number of examples, and thus are not generic. This invention provides a more versatile approach for the synthesis of small cyclic peptides, ligation of peptide segments, backbone protection and linkage of peptide to resin during solid phase peptide assembly.

SUMMARY OF THE INVENTION

This invention describes the use of a new class of auxiliaries that are readily attached to primary amines, for instance at the N-terminus of a peptide, allow for efficient acylation of the nitrogen atom, and in preferred embodiments are readily removed by photolysis. The overall outcome of the use of this auxiliary is the formation of an amide bond. As described below, this new class of auxiliaries has applications in the synthesis of linear peptides and cyclic peptides, in ligation chemistry, and as a backbone linker.

In a first aspect, the invention provides a method of synthesis of linear or cyclic peptides or C-terminal modified peptides, or of on-resin cyclisation of molecules, comprising the step of linking a compound of General Formula I



I

in which

X is oxygen or sulphur;

Y is an electron-withdrawing group;

Z is any group which allows the formation of a covalent carbon-nitrogen bond; and

R^3, R^4 and R^5 are each independently hydrogen, alkyl, substituted alkyl, aryl, substituted aryl, arylalkyl, substituted arylalkyl, heteroaryl, substituted heteroaryl, alkoxy, aryloxy, XH or Y, or a covalent linkage to a solid support.

It will be appreciated by those skilled in the art that a degree of symmetry exists in the numbering of atoms in the ring. For the purpose of this specification the numbering scheme shown in Formula I will be used.

Suitable electron-withdrawing groups Y include but are not limited to nitro, ketone, carboxylic ester, amide, nitrile, sulfonamide, sulfoxide, sulfone, sulfonate, fluoride, chloride, bromide and iodide. Other suitable groups are known to those skilled in the art. See for example March (1985).

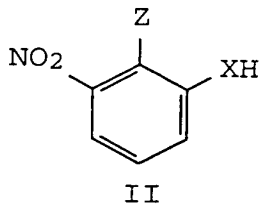
Z is suitably an aldehyde, alkylalcohol, alkylhalide, ketone, or halogenated C_{1-3}alkyl group, in which the halogen is preferably iodine, bromine or chlorine. Preferably the halogenated alkyl group is a methyl group. Suitable Z groups are well known to those skilled in the art. See for example Houben-Weil (1957).

Preferably the XH group is at position 2 or 3 and Y is at any other position; more preferably the XH group is at position 2 and Y is at any other position; most preferably XH is at position 2 and Y is at position 6.

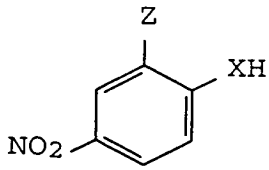
In preferred embodiments of the invention the electron-withdrawing group Y is in position 6. In particularly preferred embodiments, Y is NO_2 in position 6. In these cases, the linkage between the compound of general formula I and the amine nitrogen atom is photolabile.

Depending upon the structure of the compound of general formula I, this compound can provide a reversible auxiliary for formation of amide bonds, with enhancement of the acyl transfer, or a non-reversible auxiliary for the formation of substituted amide bonds, in which acyl transfer is enhanced, which is particularly useful in the synthesis of cyclic peptides. Thus, the compounds of

Formula II enable the synthesis of cyclic peptides, large peptides, and difficult peptides which were inaccessible by methods previously available in the art, and allow photolytic removal of the auxiliary after amide bond formation, and compounds of formula III enable the synthesis of cyclic peptides, large peptides and difficult peptides containing substituted amide bonds in which the auxiliary is not removed.



II



III

In a second aspect, the invention provides a method of synthesis of a compound selected from the group consisting of linear and cyclic peptides, large peptides with a native peptide backbone, "difficult" peptide sequences, or of backbone linkage for the synthesis of peptides, C-terminal modified peptides, or for on-resin cyclisation, comprising the step of linking a compound of General Formula I, General Formula II or General Formula III to an amine nitrogen atom. Preferably XH is in position 2 and Y is nitro in position 6. The linkage is then photolabile.

R^3, R^4 and R^5 are preferably independently selected from the group consisting of hydrogen, alkyl, aryl, heteroaryl, hydroxy, alkoxy, aryloxy, and a covalent linkage to a solid support.

In one preferred embodiment, the invention provides a method of synthesis of a cyclic peptide, comprising the steps of

a) synthesising a linear peptide to be cyclised,

b) linking an auxiliary of the invention to the desired primary amine of the linear peptide,

c) activating the desired carboxylic acid to effect cyclisation, and where necessary inducing ring contraction, and optionally

d) removing the auxiliary after complete N-acylation.

Ring contraction may be induced by known methods, including but not limited to heating or the addition of metals.

Preferably the auxiliary is of General Formula II, and is removed by photolysis.

A person skilled in the art will appreciate that the above steps can be performed on a solid support, and can be followed by cleavage of the cyclic product from the solid support, and if desired, removal of side chain protecting groups.

A person skilled in the art will also appreciate that the order of steps may be altered in order to obtain the same result. For example, activation of the C-terminal carboxylic acid can be performed in the presence of compounds of Formula I and the cyclisation performed by attaching the auxiliary to the desired amine via the Z-group.

In a second embodiment, the invention provides a method of synthesis of a large peptide with a native peptide backbone, comprising the steps of

a) synthesising a set of peptide fragments to be linked to form a large peptide,

b) linking an auxiliary of the invention to the primary amine of the first peptide fragment,

c) activating the carboxylic acid of the second peptide fragment,

d) adding the second peptide fragment to the first peptide fragment and forming a peptide bond between the two fragments, and optionally

e) removing the auxiliary after N acylation is complete.

Preferably the auxiliary is of General Formula II, and is removed by photolysis.

In a third embodiment, the invention provides a method of synthesis of a difficult peptide sequence, comprising the steps of

a) linking an auxiliary of the invention to one or more nitrogen atoms in peptide bonds of a peptide linked to a solid support,

b) synthesising the complete peptide using standard solid phase synthesis methods, and optionally

c) when synthesis is complete, removing the auxiliary.

Preferably the auxiliary is of General Formula II, and is removed by photolysis.

In a fourth embodiment, the invention provides a method of backbone linkage for the synthesis of peptides, comprising the steps of

a) using an auxiliary of the invention as a linker linking the α-nitrogen of an acid residue in the desired peptide to a solid support,

b) assembling the linear peptide using standard solid phase peptide synthesis methods, and optionally

c) removing the side chain protecting group, and/or

d) cleaving the peptide from the solid support.

The same method can be used for synthesis of C-terminal modified peptides. In this case, the carboxylic acid group of the C-terminal amino acid residue is replaced by a group such as an ester, alkylalcohol, acetal or amide. Other suitable functional carboxylic acid replacement groups are known to those skilled in the art. Preferably Y is nitro in position 6, XH is in position 2 and cleavage is performed by photolysis.

In a fifth embodiment, the invention provides a method of on-resin cyclisation of a linear peptide, comprising the steps of

a) using an auxiliary of the invention as a linker linking the α-nitrogen of an amino acid residue in the desired peptide to a solid support,

b) synthesising a linear peptide on a solid support, using standard solid phase peptide synthesis methods,

c) deprotecting the desired amine and carboxylic acid groups,

d) activating the carboxylic acid group to perform cyclisation, and optionally

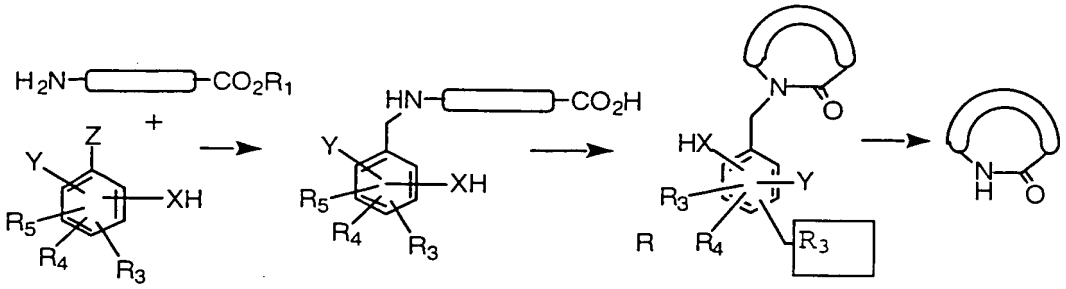
e) deprotecting amino acid side chain groups, and/or

f) cleaving the cyclic peptide from the solid support.

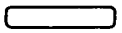
Preferably Y is a nitro group in position 6, XH is in position 2, and cleavage is performed by photolysis.

Thus the auxiliaries of the invention may be used for the following purposes:

1. To enable the synthesis of "difficult" cyclic peptides, including small cyclic peptides (up to 10 amino acid residues long). The auxiliary is attached to the primary amine, of the linear peptide, and cyclisation is performed by activating the C-terminal carboxylic acid. Optionally the auxiliary is removed after complete N-acylation.

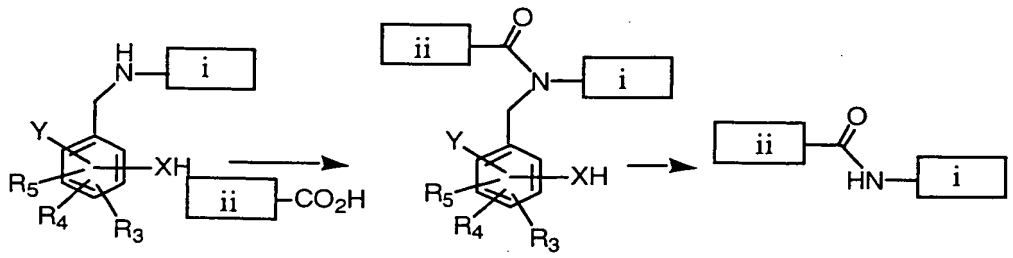


R_3, R_4 and R_5 = as described above

 = Chemical substructure, *eg.* peptide, peptidometic. These may contain a covalent link to a solid support through an amino acid side chain functionality or though an amide nitrogen atom.

R_1 = H, alkyl ester, covalent link to a solid support

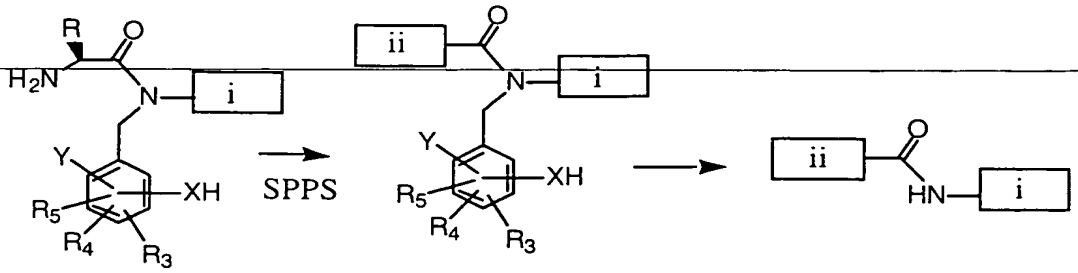
2. To enable the synthesis of large peptides. The auxiliary is attached to the primary amine of the first fragment, and the carboxylic acid of the second fragment is activated and added to the first fragment. After N-acylation is complete the auxiliary is optionally removed.



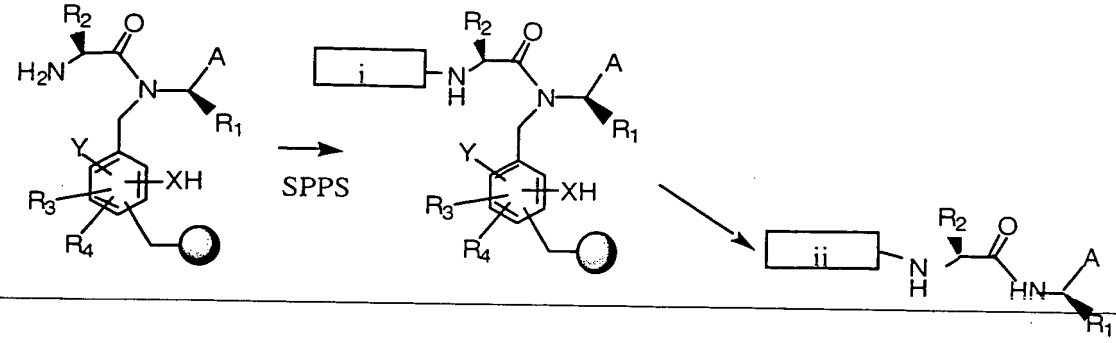
i , ii = molecular fragment, typically peptide. The molecular fragment can be covalently linked to a solid support.

3. To enable synthesis of "difficult" peptide sequences. The auxiliary can be used as a backbone

protecting group to avoid aggregation of peptides and enhance solid phase peptide assembly. After introducing the auxiliary, standard SPPS protocols are used for the synthesis of the peptide. Optionally at the end of the synthesis the auxiliary is removed by photolysis.

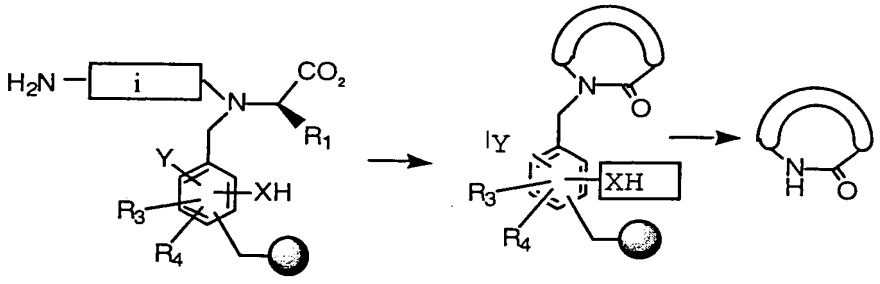


4. To enable backbone linkage for the synthesis of peptides, or C-terminal modified peptides. The auxiliary is employed as a linker to link the α-nitrogen of an amino acid residue to the resin. Standard SPPS protocols can then be employed to assemble the linear peptide. At the C-terminal residue, modified carboxylic acids can be used, such as esters, alcohols, acetals, amides or any other functional group. Alternatively, the linear peptide is cyclised on-resin, after deprotecting the N-terminal amine and the C-terminal carboxylic acids. Side chain deprotection may then be performed prior to photolytic cleavage. (A = carboxylic acid or modified acid, such as ester, acetal, amide, alcohol)



= solid support

5. To enable backbone linkage for the on-resin cyclisation of molecules. In this case the linear peptide is cyclised on-resin, after deprotection the desired amine and carboxylic acid groups from the resin-bound linear peptide synthesised as described in 4 above. Side chain deprotection may then be performed prior to photolytic cleavage.



= solid support

In a third aspect, the invention provides an auxiliary according to any one of General Formulae I, II or III, linked to a support suitable for solid phase peptide synthesis, or to a nitrogen atom of a peptide backbone. Suitable support resins are well known to those skilled in the art, and include but are not limited to functionalised polystyrene, tentagel resins, and polyethyleneglycol resins (PEG and PEGA).

The auxiliaries of the invention are suitable for use in combination with other agents useful in the synthesis of cyclic peptides, for example those described in our co-pending Australian provisional application, entitled "Synthesis of Cyclic Peptides", filed on the same day as this application.

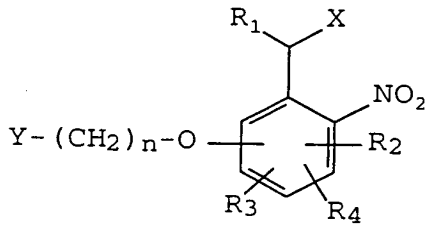
The invention also contemplates kits for use in synthesis of peptides, cyclic peptides, or organic compounds, comprising an auxiliary of the invention, or an auxiliary of the invention linked to a solid support, together with one or more other reagents for solid phase peptide synthesis.

The auxiliaries of the invention fulfil the three requirements listed above in a more versatile way, *ie.* they make a significant improvement in the yield of acylation and they can be readily introduced and optionally removed. We have significantly improved acylation rates (Step II in Scheme 8) by introducing electron-withdrawing substituents on the aromatic ring of the auxiliary. Acylation of our auxiliaries, even in hindered cases, occurs readily with mildly activated amino acids at room temperature. Furthermore, the presence of a nitro group in the ortho position to the alkylamide provides photolability. This combination of improved acylation and facile and clean removal of the auxiliary provides a novel and powerful means to generate amide bonds which is directly applicable to cyclic peptide synthesis, native ligation , the assembly of difficult peptide sequences, and backbone linking.

We have further evaluated the use of these auxiliaries in the synthesis of "difficult" cyclic peptides. We have found surprising improvements compared to the prior art methods. Where prior art methods completely failed to yield the desired product, our strategy produced target cyclic peptides in high yield and purity. The auxiliaries are expected to allow access to many classes of hitherto unknown cyclic peptides.

The auxiliary, linked to a resin, serves as a novel photolabile backbone linker for the solid phase synthesis of organic or peptidic molecules. This linker is especially valuable for the solid phase synthesis of C-terminally modified peptides and cyclic peptides.

Holmes (1997) and U.S. Patent No. 5,739,386 has described a series of photolabile compounds of the following structure:



wherein

R_1 is hydrogen, C1-C8 alkyl, aryl or arylalkyl; R_2, R_3 and R_4 are each independently hydrogen, C1-C8 alkyl, or C1-C8 alkoxy; X and Y are each independently selected from the group consisitng of halogen, -SH, -SP, -OH, -OP, $-NH_2$, -NHP, in which P is a suitable protecting or activating group, and $-NR_5R_6$ wherein R_5 and R_6 are independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, arylalkyl, substituted arylalkyl; and q is an integer from 1 to 10 and preferably from 1 to 4.

These compounds are used in solid phase chemistry to link molecules to solid supports. The linkers are stable to strong acids and bases, but are labile to UV light at around 350 nm. These compounds differ from those of our invention in at least two ways:

a) The linkers of Holmes are used only as C-terminal linkers, and are not used to facilitate amide bond formation in synthesis of cyclic peptides, peptide

ligation or synthesis of difficult sequences. They are not used to link the peptide backbone to a solid support.

b) None of the compounds described by Holmes carries a hydroxy or thiol substituent on the aromatic ring, and indeed such a substituent would have been undesirable for the purposes described by Holmes. The hydroxy-group on the aromatic ring in our compounds is crucial to enable formation of difficult amide bonds.

It will be clearly understood that for the purposes of this specification reference to solid phase peptide synthesis are to be understood to include reference to methods of synthesis of peptidomimetic compounds.

For the purposes of this specification it will also be clearly understood that the word "comprising" means "including but not limited to", and that the word "comprises" has a corresponding meaning.

For the purposes of this specification, the term "monomer" includes compounds which have an amino and carboxy terminus separated in a 1,2, 1,3, 1,4 or larger substitution pattern. This includes the 20 naturally-occurring α-amino acids in either the L or D configuration, the biosynthetically-available amino acids not usually found in proteins, such as 4-hydroxy-proline, 5-hydroxylysine, citrulline and ornithine; synthetically-derived α-amino acids, such as α-methylalanine, norleucine, norvaline, Cα- and *N*-alkylated amino acids, homocysteine, and homoserine; and many others as known to the art. It also includes compounds that have an amine and carboxyl functional group separated in a 1,3 or larger substitution pattern, such as–β-alanine, γ-amino butyric acid, Freidinger lactam (Freidinger *et al*, 1982), the bicyclic dipeptide (BTD) (Freidinger *et al*, 1982; Nagai and Sato, 1985), amino-methyl benzoic acid (Smythe and von Itzstein, 1994), and others well known to the art. Statine-like isosteres, hydroxyethylene isosteres, reduced amide bond isosteres, thioamide isosteres, urea isosteres, carbamate isosteres, thioether isosteres, vinyl isosteres

and other amide bond isosteres known to the art are also useful for the purposes of the invention. Thus the word "peptide" as used herein encompasses peptidomimetic compounds. Optionally the peptide may be protected with one or more protecting groups of the type used in the art (see for example Bodanszky, M., (1984), *"Principles of Peptide Synthesis"*, Springer-Verlag, Heidelberg).

The solid support may be of any type used for solid phase synthesis of peptides, peptidomimetics, oligonucleotides, oligosacharides or organic molecules. The solid support may be in the form of a bead, a pin or another such surface which is suitable for use in solid phase synthesis. A wide variety of suitable support materials are known in the art. See for example Meldal (1997). Commercially-available polystyrene supports, including aminomethyl-polystyrene, benzhydrylaminepolystyrene, polyethyleneglycol-polystyrene are especially suitable.

Coupling methods to form peptide bonds are well known to the art. See for example Albericio and Carpino (1997).

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described in detailed by way of reference only to the following non-limiting examples.

Abbreviations used herein are as follows:

AcOH	Acetic acid
BOC	N_{α}-tert-butoxycarbonyl
BOP	Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium-hexafluorophosphate
^{t}Bu	tert-Butyl
calcd	calculated
DCM	dichloromethane
DIEA	diisopropylethylamine
DMF	N,N-dimethylformamide

eq	equivalent
ES-MS	Electrospray Mass Spectrometry
LC/MS	Liquid Chromatoraphy linked to Mass Spectrometry
Fmoc	9-Fluorenylmethyloxycabonyl-
HBTU	O-benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate
HF	anhydrous hydrogen fluoride
NMR	Nuclear magnetic resonance
ONBS	*o*-nitrobenzene-sulfonamide
Pam	Phenylacetamidomethyl
PMC	2,2,5,7,8-pentamethylchroman-6-sulfonyl
RP-HPLC	Reversed phase high-performance liquid chromatography
RT	room temperature
SPPS	Solid phase peptide synthesis
TFA	trifluoroacetic acid
UV	Ultraviolet

Materials and Methods

Chlorotrityl resin (sv = 0.92 mmol/gr) was purchased from PepChem (Tubingen, Germany). All Wang resins and N_{α}-*tert*-butoxycarbonyl (BOC)-L-amino acids were peptide synthesis grade purchased from Auspep (Melbourne Australia), Novabiochem (San Diego) or Peptide Institute (Osaka, Japan). Pam resins were purchased from Applied Biosystems (Foster City, CA). Dichloromethane (DCM), diisopropyl-ethylamine (DIEA), N,N-dimethylformamide (DMF), and trifluoroacetic acid (TFA) were obtained from Auspep (Melbourne, Australia). p-Cresol, p-thiocresol, 3-nitrophenol, polyphosphoric acid, hexamethylenetetramine were purchased from Aldrich or Fluka (Sydney, Australia). HPLC grade acetonitrile was purchased from Millipore-Waters (Sydney, Australia). 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate and Benzo-triazole-1-yl-oxy-tris-(dimethylamino)-phosphonium-hexafluoro-phosphate was purchased from Richelieu Biotechnologies (Quebec, Canada). Deionised water was used throughout and

was prepared by a Milli-Q water purification system (Millipore-Waters). Screw-cap glass peptide synthesis reaction vessels (20 mL) with sintered glass filter frit were obtained from Embell Scientific Glassware (Queensland, Australia). Argon, helium and nitrogen (all ultrapure grade) were from BOC Gases (Queensland, Australia).

^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian 300 MHz Gemini in CD_3OD, and chemical shifts are reported in parts per million (ppm) downfield from $(CH_3)_4Si$.

Reversed phase high-performance liquid chromatography (RP-HPLC) was performed on a Waters 600E solvent delivery system equipped with a 484 absorbance detector at 214 nm or 230 nm (Applied Biosystems Inc.) or on a Hewlet Packard HP1100 system equipped with Diode-Array detector. HPLC data were collected using Turbochrome or HPLC 2D Chemstation software. RP-HPLC was performed on a Zorbax microbore (SB-C18, 2.1 mm x 5 cm) column, a Vydac C-18 analytical (5 mm, 0.46 cm x 25 cm) column or a Vydac preparative C-18 (10 mm, 2.2 cm x 25 cm) column. Chromatographic separations were achieved using linear gradients of buffer B in A (A = 0.1% aqueous TFA; B = 90% CH_3CN, 10% H_2O, 0.09% TFA) at a flow rate of 0.25 mL/min (microbore), 1 mL/min (analytical) and 8 mL/min (preparative).

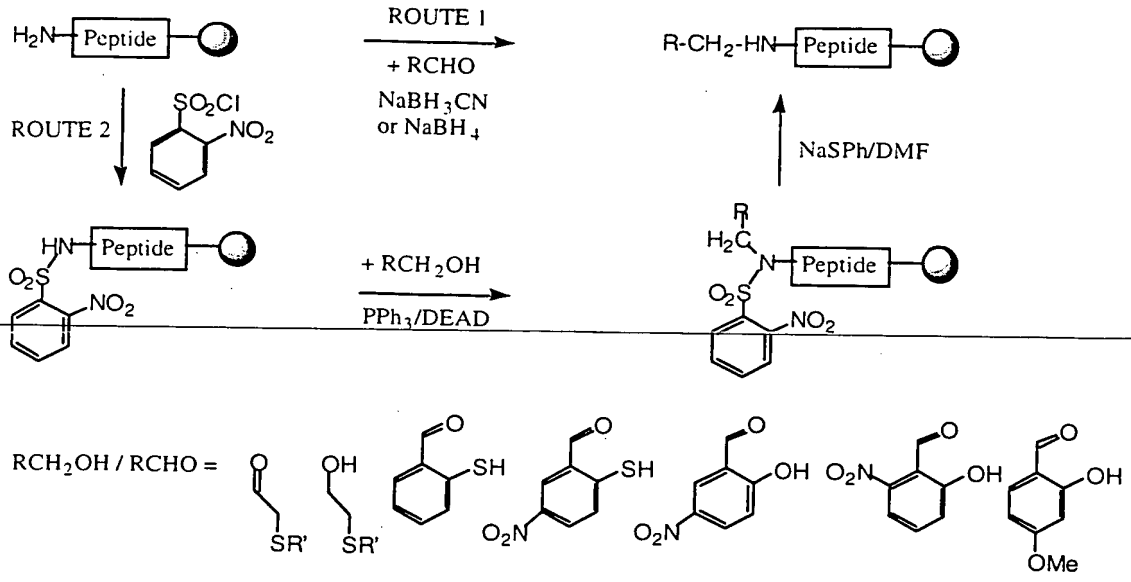
Mass spectra were acquired on a PE-Sciex API-III triple quadrupole mass spectrometer equipped with an Ionspray atmospheric pressure ionization source. Samples (10 µL) were injected into a moving solvent (30 µL/min; 50/50 CH_3CN/0.05% TFA) coupled directly to the ionisation source via a fused silica capillary interface (50 mm i.d. x 50 cm length). Sample droplets were ionized at a positive potential of 5 kV and entered the analyser through an interface plate and subsequently through an orifice (100-120 mm diameter) at a potential of 80 V. Full scan mass spectra were acquired over the mass range of 400 to 2000 Daltons with a scan step size of 0.1 Da. Molecular

masses were derived from the observed m/z values using the MacSpec 3.3 and Biomultiview 1.2 software packages (PE-Sciex Toronto, Canada). Calculated theoretical monoisotopic and average masses were determined using the MacBiospec program (PE-Sciex Toronto, Canada). LC/MS runs were carried out using a linear gradient on a 140B ABI dual syringe pump solvent delivery system and a Zorbax reversed phase C-18 (SB, 2.1 mm x 5 cm)) column at a flow rate of 150 µL/min. Samples (typically 5 µL of 1 mg/mL solution) were loaded directly on the column and the eluent directly connected to the mass spectrometer via a 30 cm, 75 mm i.d. fused silica capillary. The application of Turbo Ionspray™ (5 L/min N2 at 500°C) allowed the introduction of the total eluent without splitting and loss in sensitivity. Acquisition parameters were as described above.

Examples 1 to 5 - Introduction of the Auxiliaries

Our synthetic approach for introducing the auxiliary is depicted in Scheme 9. In brief, there are two reported pathways that provide access to the N-substituted linear peptides:

1. A first, and most common route, makes use of a reductive alkylation of the N-terminal primary amine with the aldehyde. Initially the aldehyde is added in excess to the resin-bound peptide. After imine formation is complete, as assessed by ninhydrin reaction, a solution of $NaBH_4$ in DMF/MeOH (2/1) or $NaBH_3CN$ in DMF/MeOH (1/1) containing 5% acetic acid is added to reduce the imine and generate the -CH2- link between the auxiliary and the amine.



Scheme 9

Introduction of the auxiliaries

2. In a second route an *o*-nitrobenzene-sulfonamide (ONBS) is prepared from the corresponding sulfonylchloride and the amine. A Mitsunobu-type alkylation, using the auxiliary alcohol, establishes the link with the amine. Finally the ONBS-group is removed using PhSNa in DMF. This three-step process is also known as the Fukuyama reaction.

Example 1 Synthesis of HS-$(CH_2)_2$-Tyr-Arg-Phe-Gly-OH

Synthesis was performed on Fmoc-Gly-WANG resin (0.36 mmol/g). The tetrapeptide Tyr-Arg-Phe-Gly was assembled using stepwise Fmoc-SPPS, with alternating HBTU coupling and piperidine deprotection as follows:

Coupling

2mmol Fmoc-amino acid (4 equivalents) were dissolved in DMF (4mL) and 2 mmol HBTU added to the solution together with DIEA (400 µL). After 1 minute preactivation the solution was added to the amino-resin. The reaction was left for 10 minutes and ninhydrin test

performed on a small resin sample to measure the amount of remaining free amine. If the measured amount of free amine was more than 0.5%, the reaction was left longer (30 minutes) and if necessary repeated. The resin was then washed several times with DMF.

Deprotection

The Fmoc protected resin (0.5 mmol) was treated with a solution of Piperidine/DMF (1/1) (4mL) for 2 minutes. The resin was drained and the piperidine treatment repeated. The resin was then washed several times with DMF before the next coupling step was commenced.

The Tyr(Bu)-Arg(PMC)-Phe-Gly-WANG resin (1 g) was then treated with S-(p-methylbenzyl)-2-mercapto-acetaldehyde (58 mg; 0.32 mmol, Bitan *et al*, 1997) dissolved in MeOH/DMF/AcOH (47/47/5) (6 mL). After 5 min stirring 60 mg of $NaBH_3CN$ was added and the mixture left for 60 minutes. The reductive alkylation step was then repeated once more to ensure complete reaction. The resin was washed several times with DMF/MeOH, MeOH/DCM and DCM and finally air dried.

HF cleavage

1 g of resin was treated with 1 mL of *p*-cresol and 9 mL HF at 0°C for 1 hour. The HF was evaporated *in vacuo* and the residue triturated with cold diethylether (20 mL). The ether was filtered off and the precipitated dissolved in a small amount of water. The solution was then loaded directly on to an HPLC column (Vydac, C18 reverse phase 2.1 x 25 cm) for HPLC purification of the products (buffer A:water, 0.1% TFA; buffer B: acetonitrile/water 1/1, 0.09% TFA) (100% A to 80% B in 60 min). The $HS-(CH_2)_2-NH-CH(CH_2PhOH)-CO-Arg-Phe-Gly-OH$ was isolated and lyophilised, yielding 70 mg of white powder (45% yield), Mr 601.5, calcd for $C_{28}H_{39}N_7O_6S$:601.27.

Example 2 Synthesis of N-(5-nitro-2-mercaptobenzyl)-Tyr-Arg-Phe-Gly-OH

Tyr(Bu)-Arg(PMC)-Phe-Gly-WANG resin was prepared as described in Example 1.

120 mg of 2,2'-dialdehyde-4,4'-dinitro-[diphenyldisulfide] (Fries and Brothuhn, 1923) (0.33 mmol) was dissolved in MeOH/DMF/AcOH (47/47/5) (3 mL) and the solution added to the resin. After 5 minutes, 100 mg $NaBH_3CN$ (1.6 mmol) was added and the solution stirred for 15 hours. The resin was washed with DMF (3x) and MeOH/DCM 1/1 (3x).

TFA cleavage

700 mg of resin was treated with 10 mL TFA/H_2O (95/5) for 1 hour at room temperature. The TFA was removed *in vacuo* and the residue dissolved in HPLC buffers A/B 1/1 (5 mL). The solution was then loaded directly on to an HPLC column and purification of the product performed as in Example 1. 25 mg N-(5-nitro-2-mercaptobenzyl)-Tyr-Arg-Phe-Gly-OH were obtained from lyophilisation (20% yield), Mr 708.4 (calcd for $C_{33}H_{40}N_8O_8S$: 708.27).

Example 3 Synthesis of HS-$(CH_2)_2$-Ala-Phe-Leu-Pro-Ala-OH

Ala-Phe-Leu-Pro-Ala-WANG resin was prepared starting from Fmoc-Ala-WANG resin (0.44 mmol/gram) using standard Fmoc-SPPS protocols, with HBTU coupling and piperidine deprotection as described in Example 1. To 500 mg of this resin , a solution of 300 mg *o*-nitrobenzenesulfonylchloride in DMF (4 mL) containing DIEA (200 µL) was added. After 30 minutes, the resin was drained and washed with DMF (3x). The resin was mixed with a solution of S-(p-methylbenzyl)-2-mercaptoethanol (270 mg, 1.5 mmol) in DCM (5mL). Triphenylphosphine (393 mg, 1.5 mmol) and diethylazodicarboxylate (DEAD, 261 mg, 1.5 mmol) were premixed in DCM (5 mL). After 1 minute, the solution was added to the resin and the reaction left for 30 minutes. The resin was washed with DCM (3x) and

DMF(3x). The resin was further treated with a solution of NaSPhe (200 mg, 1.5 mmol) in DMF (4 mL) for 30 minutes. The resin was washed with DMF (3x) and MeOH/DCM (3x) and air dried.

HF cleavage

500 mg of resin was cleaved using HF/*p*-cresol/*p*-thiocresol (9/1/1) (10 mL) (1 hour at 0°C) and was worked up as described in Example 1. The crude residue was dissolved in buffers A/B (1/1) and purified on HPLC yielding HS-$(CH_2)_2$-NH-CH(CH_3)-CO-Phe-Leu-Pro-Ala-OH (25 mg, 22% yield). Mr : 577.1 (calc for $C_{28}H_{43}N_5O_6S$: 577.29)

Example 4 Synthesis of N-(2-hydroxy-5-nitrobenzyl)-Ala-Phe-Leu-Pro-Ala-OH

Fmoc-Ala-trityl resin (0.4mmol/gr) was first prepared from trityl resin (0.96 mmol/gr) using protocols provided by Pepchem (Tubingen, Germany). Ala-Phe-Leu-Pro-Ala-Trityl resin was assembled using standard Fmoc SPPS protocols, as in Example 1. This resin (0.5 gr) was further treated with a solution of 2-hydroxy-5-nitrobenzaldehyde (115 mg, 0.7 mmol) and AcOH (20 µL) in DMF (2 mL). After 5 minutes the resin was drained and a second aldehyde treatment was performed. The resin was drained, and washed copiously with DMF until eluent was colourless. A solution of $NaBH_4$ (150 mg, 4 mmol) in DMF/MeOH 3/1 (4 mL) was added and the resin stirred for 10 minutes. The resin was drained, washed with DMF/MeOH 1/1, DCM/MeOH 1/1 and DCM and air dried.

TFA cleavage

The resin was treated with DCM (10mL) and TFA (100 µL) for 1 hour. The solution was evaporated, buffer B (3 mL) was added and the resin filtered off. The solution was loaded directly on to a preparative HPLC column and HPLC purification performed using a 2% gradient (from 90% A

to 10% A in 40 minutes). After lyophilisation N-(2-hydroxy-5-nitrobenzyl)-Ala-Phe-Leu-Pro-Ala-OH (114 mg) was isolated as a white powder (85% yield), Mr: 668.2 (calcd for $C_{33}H_{44}N_6O_9$: 668.32).

Example 5 Synthesis of N-(2-hydroxy-6-nitrobenzyl)-Ala-Phe-Leu-Pro-Ala-OH

The linear peptide was synthesised on trityl resin as described in example 4, but employing 2-hydroxy-6-nitrobenzaldehyde (Harayama *et al*, 1994). After lyophilisation N-(2-hydroxy-6-nitrobenzyl)-Ala-Phe-Leu-Pro-Ala-OH (85 mg) was isolated as a white powder (63% yield), Mr 668.2, calcd for $C_{33}H_{44}N_6O_9$: 668.32).

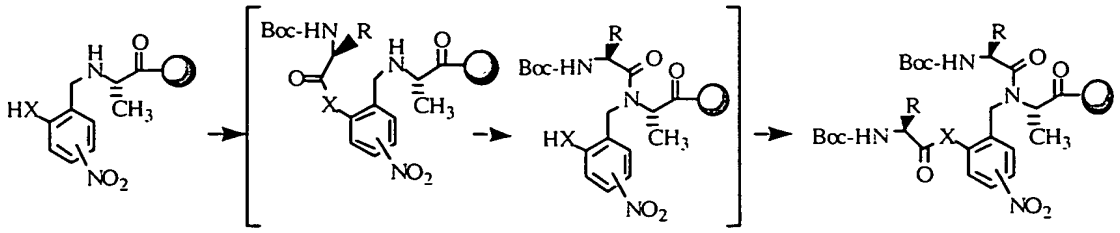
Example 6 Acylation Rate of the Arylamine

The most important factor limiting the use of the prior art methods was the *O*-to-*N* or *S*-to-*N* acyl transfer step. One of the parameters which we envisaged would have a large impact on the rate of acyl transfer was the activation of the intermediate oxy- or thioester bond. With this in mind we focussed on the following auxiliary modifications:

- Introduction of a nitro substituent on the aromatic ring of the auxiliary. Nitrophenyl esters react more readily with nucleophiles when compared to phenyl esters.

- Thio-esters versus oxy-esters. In previous work in our laboratory we found that under the same conditions both esters hydrolyse at the same rate. We have included both phenols and thiophenols in our set of N-substituents.

To compare the rate of acyl transfer we carried out the reactions shown in Scheme 10.



Starting Material Monoacylated material Diacylated materia

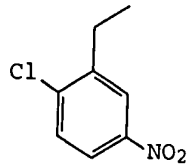
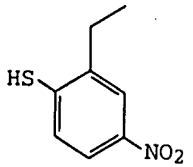
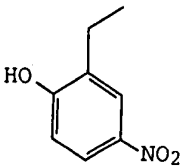
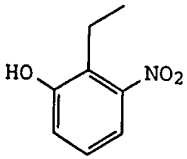
Scheme 10
Acylation experiments

A tripeptide (Ala-Gly-Phe) was assembled on WANG-resin, and the selected range of aldehydes introduced via reductive amination. Each N-substituted tripeptide was then subjected to acylation by mixing the resin with a solution of HBTU-activated Boc-amino acid (Boc-Ala-OH, Boc-Phe-OH or Boc-Val-OH). The general reaction pathway was as follows: acylation of the N-substituted tripeptide occurs initially on the phenol oxygen (or sulfur) atom. The acyl group subsequently migrates to the nitrogen atom. The liberated phenol (or thiophenol) functionality was then acylated a second time.

We included as a control the 2-chloro-4-nitrobenzyl substituent. Due to the absence of the alcohol we expected that acylation directly on to the secondary amine, will be much slower then for the phenol/thiophenol cases. The results were summarized in Table 1.

Table 1

Distribution of *N*-terminal acylation products on the N-[auxiliary]-AGF sequence

		N-terminal Acylation Products (%)[a]								
		Alanine			Phenylalanine			Valine		
Auxiliary	t (min)	unreacted	monoacylated	diacylated	unreacted	monoacylated	diacylated	unreacted	monoacylated	diacylated
	30	>98	ND	ND	>98%	ND	ND	>98%	ND	ND
	1	33	40	27	10	57	33	18	58	24
	10	5	77	18	4	12	84	8	19	73
	60	3	60	37	ND	11	89	3	9	88
	1	4	8	88	2	17	81	26	47	27
	10	2	2	96	ND	2	98	4	19	77
	60	ND	1	99	ND	<1	99	2	15	83
	1	ND	35	65	4	21	75	48	46	6
	10	ND	27	73	ND	18	82	7	63	30
	60	ND	16	84	ND	21	79	4	14	82

[a]ND indicates product not detected.

The following conclusions can be drawn from these results:

1. The presence of the alcohol/thiol function at the ortho position of the aromatic ring dramatically increases the rate of acylation of the substituted amine. Under the same acylation conditions, the ortho-chloro "auxiliary" did not undergo acylation at the secondary amine site.

2. The presence of an alcohol/thiol function in combination with a nitro substituent on the aromatic ring ensures very rapid acylation of the secondary amine. For most of the cases studied >70% diacylated product was formed after 10 minutes. Acylation of the 2-hydroxy-6-nitro-derivative with Boc-Valine required 60 minutes to achieve 82% diacylation. In other words, acylation at the nitrogen atom was >80% complete after 60 min. when Boc-Valine was used. As a comparison, in a very similar study acylation of the alpha-nitrogen atom of N-[2-hydroxy-4-methoxybenzyl]-alanine with Fmoc-valine was still incomplete after 24 hours. In the case of acylation of the 2-mercapto-5-nitro-derivative with Boc-Alanine, diacylation only reaches 37% after 60 min.; from comparison with the other results we ascribe this to hydrolysis of the highly reactive and less hindered ester bond in the diacylated product during work-up.

3. The position of the nitro-group on the ring does not seem to play a major role, although small differences can be observed. Substituting oxy-ester for thioester does not significantly alter the rate.

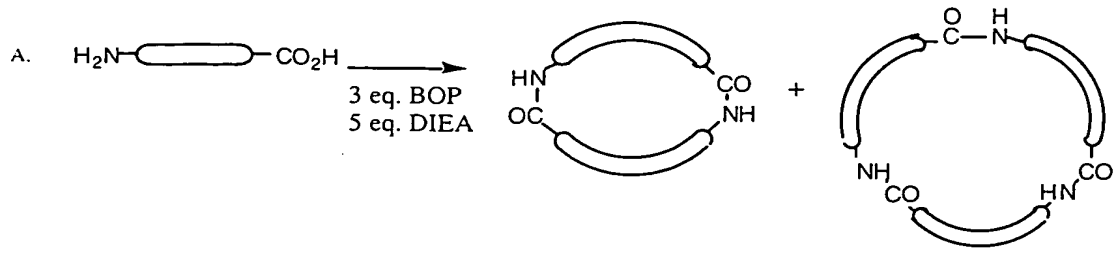
Experimental

The Ala-Gly-Phe sequence was assembled on Wang resin (0.1 mmol, Novabiochem) using standard Fmoc/HBTU protocols. Auxiliaries were introduced as described in Examples 2, 4 and 5. Each resin was then distributed into three portions in a separate reaction vessels, and swelled in DMF for 10 min. Three equivalents of Boc-Ala-OH,

Boc-Phe-OH, or Boc-Val-OH were coupled to Ala-Gly-Phe-resin using 0.99 equivalents of HBTU with 4 equivalents of DIEA. Samples (10-15 mg) were removed after 1 minute, 10 minutes and 60 minutes, immediately drained, washed with 1:1 DCM:MeOH, and dried. Samples were then cleaved with 250 mL of 97% TFA at room temperature for 1 h. The TFA was evaporated with a stream of nitrogen and the product dissolved in 50% B (100 mL). The sample was centrifuged and the supernatant collected, then analysed by RP-HPLC and ES-MS or LC/MS. The relative yields of unreacted starting material, monoacylated and diacylated product for each experiment were calculated by integrating the HPLC signals in the corresponding chromatogram.

Example 7 Peptide Cyclisation Through Ring Contraction

H-Ala-Phe-Leu-Pro-Ala-OH was a recently reported example of a sequence which is difficult to cyclise (Schmidt and Langner, 1997). When subjected to cyclisation conditions, dimer and higher oligmers were generated, but no target cyclocpentapeptide was formed. We have employed this linear peptide to probe our methodology and compare it with the prior art methods. In the following set of experiments, summarized in Scheme 11, we demonstrate that this Ala-Ala amide bond in the monocycle was not accessible from this linear peptide using prior art methodologies, but was accessible using our photolabile auxiliaries.



Scheme 11

Cyclisation of a difficult sequence

Scheme 11A - Cyclisation of Ala-Phe-Leu-Pro-Ala

Cyclisation of this peptide under standard cyclisation conditions (1 mM in DMF, 3eq. BOP, 5 eq. DIEA, 3h) produced only cyclic dimer and cyclic trimer. This was in agreement with the previously reported results.

Scheme 11B - Cyclisation of HS-$(CH_2)_2$-Ala-Phe-Leu-Pro-Ala

The oxyethanethiol auxiliary group used for intramolecular "native ligation" has only been successfully applied to X-Gly cyclisation sites. We examined the alkylthiol ligation approach by synthesising N-(HS-$(CH_2)_2$)-Ala-Phe-Leu-Pro-Ala and subjecting it to the standard cyclisation conditions. The isolated product from this reaction was the cyclic thioester. This was confirmed by hydrolysing the thioester in NH_4HCO_3 to generate disulfides of the linear peptide amides or acids. Attempts to force acyl transfer by heating the ester in DMF/DIEA, or in aqueous buffers failed. The ester either remained unchanged, or hydrolysed to the linear peptide.

Scheme 11C - Cyclisation of N-(5-nitro-2-hydroxybenzyl)-Ala-Phe-Leu-Pro-Ala-OH

The thioester described above failed to undergo acyl transfer, even under forcing conditions. In contrast, our approach was to promote acyl transfer by introducing electron withdrawing substituents on the auxiliary group. We initially employed the commercially available 5-nitro-2-hydroxybenzaldehyde to synthesise the N-(5-nitro-2-hydroxybenzyl)-Ala-Phe-Leu-Pro-Ala-OH. Optimal results of cyclisation were obtained when excess DIEA was added after 3h of cyclisation at RT and the mixture either left standing for another 24 h, or heated for 1 hour. The cyclic amide was isolated in 51% yield. A ^1H NMR absorption for a phenolic proton at 11.5 ppm confirmed that the product contained a free hydroxy substituent, and thus did not have the ester structure but rather the target cyclic amide structure.

Scheme 11D - Synthesis of cyclo-[Ala-Phe-Leu-Pro-Ala] using a photolabile auxiliary

The previous example illustrates that the introduction of a nitro group facilitates the acylation of

the secondary amine. It has further been reported that *o*-nitrobenzyl substituents on amines or amides can be removed photolytically. In this experiment we have employed 6-nitro-2-hydroxybenzaldehyde as above. Thus N-(6-nitro-2-hydroxybenzyl)-Ala-Phe-Leu-Pro-Ala-OH was synthesised and cyclised as for example C yielding cyclic amide. The auxiliary in the isolated cyclic product was then successfully removed by UV-photolysis at about 360 nm to yield the target cyclo-[Ala-Phe-Leu-Pro-Ala] in the all L configuration. The product coeluted with an independently synthesised sample.

Experimental

A. Cyclisation of Ala-Phe-Leu-Pro-Ala

Ala-Phe-Leu-Pro-Ala was synthesised on chorotrityl resin as described in Example 4. The peptide was cleaved from the resin and purified as described in Example 4, and was isolated in 90% yield. Cyclisation of this linear unsubstituted peptide was performed as follows: peptide (1 mg, 1.6 µmol) was dissolved in DMF (1.9 mL), containing BOP (2.6 mg, 3 eq) and DIEA (1.7 µL, 5 eq). The reaction was stirred for 3 hours and solvent evaporated *in vacuo*. The residue was dissolved in buffers A/B 1/1 (0.5 mL), and analysed by analytical HPLC and LCMS. Only dimer (Mr: 998.7, calcd for $C_{52}H_{74}N_{10}O_{10}$: 998.56) and trimer (Mr: 1498.2, calcd for $C_{78}H_{111}N_{15}O_{15}$: 1497.84) were found in the product mixture.

B. Cyclisation of HS-$(CH_2)_2$-Ala-Phe-Leu-Pro-Ala

HS-$(CH_2)_2$-Ala-Phe-Leu-Pro-Ala was synthesised as described in Example 3, and subjected to the following cyclisation conditions: linear peptide (10 mg of the TFA salt, 0.014 mmol) was dissolved in DMF (17 mL) containing BOP (23 mg, 3 eq) and DIEA (15 µL, 5 eq). The reaction was stirred for three hours, the solvent was evaporated and the residue dissolved in buffers A/B 1/1 (2 mL). The solution was subjected to preparative HPLC (Vydac C18 reverse phase,

from 90% A to 10% A in 40 minutes). The major product was the monocyclic thioester (3.4 mg, 45% yield): Mr: 559.3, calcd for $C_{28}H_{41}N_5O_5S$: 559.28).

Hydrolysis of the thioester

The product was dissolved in aqueous ammoniumbicarbonate buffer (0.1 M, pH 8) and left standing for 10 hours at 60°C. The thioester hydrolysed to the C-terminal amide and the C-terminal acid. Under the mild base conditions these thiol-products oxidised to form disulfides of the linear peptides which were characterised by ESMS. [S-$(CH_2)_2$-NH-CH(CH_3)-CO-Phe-Leu-Pro-Ala-NH_2]$_2$ Mr: 1150.8, calcd for $C_{56}H_{86}N_{12}O_{10}S_2$: 1150.6, [S-$(CH_2)_2$-NH-CH(CH_3)-CO-Phe-Leu-Pro-Ala-NH_2]-S-$(CH_2)_2$-NH-CH(CH_3)-CO-Phe-Leu-Pro-Ala-OH Mr : 1151.8, calcd for $C_{56}H_{85}N_{11}O_{11}S_2$: 1151.6, [S-$(CH_2)_2$-NH-CH(CH_3)-CO-Phe-Leu-Pro-Ala-OH]$_2$ Mr: 1152.8, calcd for $C_{56}H_{84}N_{10}O_{12}S_2$: 1152.6.

Attempt at acyl transfer

The cyclic thioester was dissolved in DMF (10^{-3}M) or dioxane (10^{-3}M), containing 10 eq DIEA or 10 eq. DBU, and the solution was heated to 65°C for 24 hours. The thioester remained unchanged after this time (HPLC/MS).

C. Cyclisation of N-(5-nitro-2-hydroxybenzyl)-Ala-Phe-Leu-Pro-Ala-OH

We synthesised N-(5-nitro-2-hydroxybenzyl)-Ala-Phe-Leu-Pro-Ala-OH as described in Example 4 and subjected it to the following cyclisation conditions: linear peptide (30 mg of the TFA salt, 0.038 mmol) was dissolved in DMF (22.5 mL) containing BOP (19.2 mg, 0.043 mmol, 1.1 eq) and DIEA (15.2 µL, 0.087 mmol, 2.2 eq) and the reaction stirred for 3 hours. After this time excess DIEA (0.9 mL) was added and the solution heated to 65°C for 1 hour. The solvent was then evaporated and the residue subjected to preparative HPLC as described above. The major product was isolated and characterised as the cyclo-[N-(5-nitro-2-

hydroxy-benzyl)-Ala-Phe-Leu-Pro-Ala-] (12.5 mg, 0.019 mmol, 51% yield) : ES-MS Mr: 650.2, calcd for $C_{33}H_{42}N_6O_8$, 650.3 (monoisotopic). ^1H NMR (500 MHz, DMSO-d_6, ppm) δ 11.5 (s, 1H, aromatic OH), 8.40 (d, 1H, N*H*Leu), 8.02 (dxd, 1H, *H*-ar), 7.70 (d, 1H, *H*-ar), 7.4 (d, 1H, *H*NPhe), 7.20-7.30 (m, 5H, *H*-Phe), 6.99 (d, 1H, *H*-ar), 6.54 (d, 1H, *H*-NAla), 5.00 (s, 1H, ArCH*h*N-), 4.91 (m, 1H, α-Ala^5), 4.75 (q, 1H, α-Ala^1), 4.59 (m, 1H, α-Phe), 4.50 (m, 1H, α-Leu), 4.27 (t, 1H, α-Pro), 3.88 (d, 1H, ArCH*h*N-), 3.62 (m, 1H, δ-Pro), 3.37 (m, 1H, δ-Pro), 2.97 (m, 1H, β-Phe), 2.82 (m, 1H, β-Phe), 2.04 (m, 2H, β-Pro), 1.88 (m, 1H, γ-Pro), 1.73 (m, 1H, β-Leu), 1.65 (m, 1H, γ-Pro), 1.44 (m, 1H, γ-Leu), 1.33 (m, 1H, β-Leu), 1.24 (d, 3H, β-Ala^5), 0.91 (d, 3H, β-Ala^1), 0.85 (m, 6H, δ-Leu). ^{13}C NMR (75 MHz, DMSO-d_6, ppm) δ 172.61, 170.34, 170.07, 169.95, 169.47, 160.40, 139.73, 136.88, 129.31, 128.14, 126.50, 125.72, 124.21, 122.65, 115.00, 61.04, 56.50, 55.74, 48.70, 46.31, 44.34, 41.37, 38.28, 31.30, 24.20, 22.81, 22.68, 21.17, 18.97, 15.35.

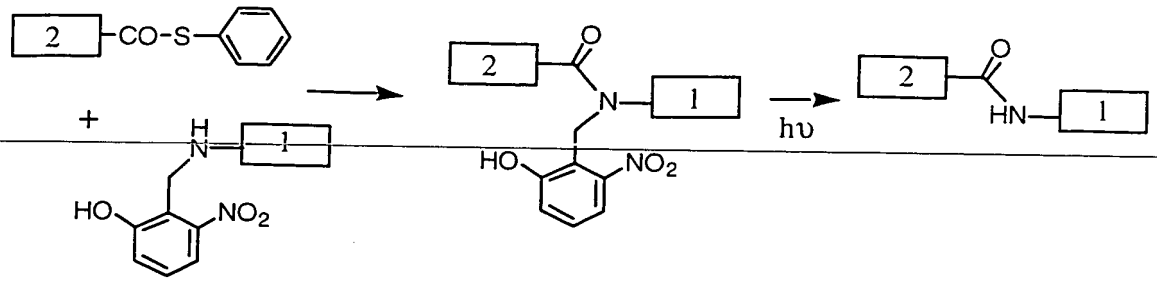
D. Synthesis of cyclo-[Ala-Phe-Leu-Pro-Ala]

a. In a two step process:

Cyclisation of N-(6-nitro-2-hydroxybenzyl)-Ala-Phe-Leu-Pro-Ala-OH:

Cyclisation was performed as described for the N-(5-nitro-2-hydroxybenzyl)-Ala-Phe-Leu-Pro-Ala-OH. The major product was isolated and characterised as the cyclo-[N-(2-hydroxy-6-nitrobenzyl)-Ala-Phe-Leu-Pro-Ala-] (7 mg, 28% yield) : ES-MS *Mr* 650.2, calcd for $C_{33}H_{42}N_6O_8$: 650.3061. ^{13}C NMR (75 MHz, CD_3OD, ppm) δ 178.07, 176.95, 174.54, 174.32, 173.72, 159.11, 153.19, 140.41, 131.99, 129.96, 129.54, 127.57, 121.18, 116.57, 62.75, 60.67, 58.55, 54.05, 51.15, 44.54, 43.41, 34.85, 33.67, 25.03, 24.13, 22.30, 21.31, 15.49, 13.89.

The oxyethanethiol auxiliary approach has been applied to these ligation sites (Canne *et al*, 1996), and was only successful in the first, non-hindered, case.



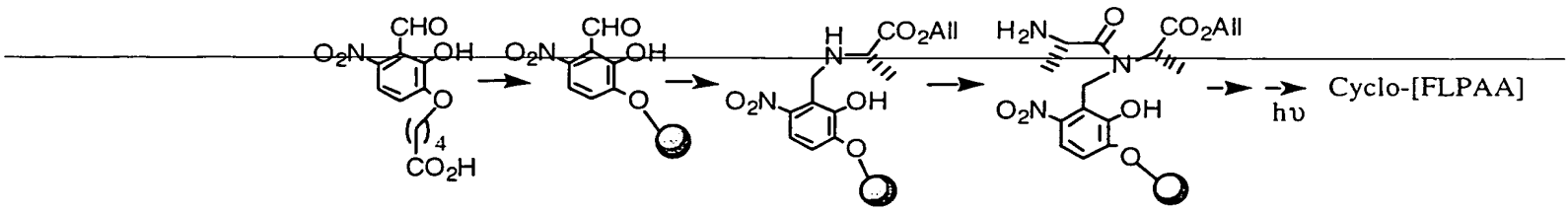
Peptide 2		Peptide 1	Product
LYRAG	+	GAGPA	LYRA**GG**AGPA
LYRAF	+	AARHT	LYRA**FA**ARHT

The first peptide segments (peptide 1) are assembled using standard *in situ* neutralisation protocols and the auxiliary is introduced as described in Examples 1 to 5. Standard HF cleavage and side chain deprotection provides the first unprotected peptide segment. The second peptide segments (peptide 2), containing a thiophenylester at the C-terminus, are synthesised as described before (Canne *et al*, 1996).

To optimise the ligation conditions the following experiments are then performed : peptide 1 and peptide 2 are dissolved in DMF at 1 mM, 10 mM and 100 mM concentration and 2 or 5 equivalents of DIEA added. Progress of the reaction is monitored for each experiment by HPLC and LCMS analysis at different time intervals. Several other solvent systems are tested, such as DMSO/DIEA, and aqueous buffers (pH ranging from 4 to 8) (no DIEA).

Example 9 Backbone Linker

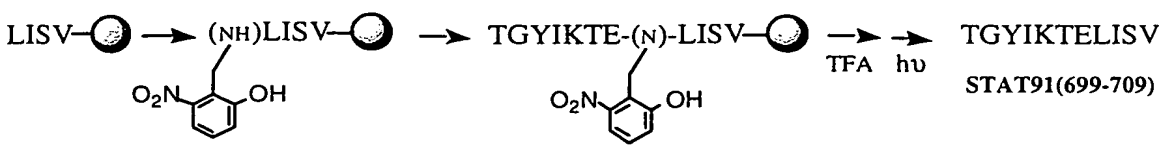
In this experiment we illustrate how we accommodate photolabile backbone linking using this auxiliary approach for the solid phase synthesis of cyclic peptides.



2,3-dihydroxy-6-nitrobenzaldehyde (Perez *et al*, 1992) is treated with 1 equivalent bromovaleric acid and 1 equivalent $KHCO_3$ in acetone. The resulting acid is linked to aminomethylated polystyrene. Alanine allyl ester is attached to the resin by reductive amination and the resulting secondary amine acylated with Boc-Ala-OH as described in Example 6. The linear peptide (Phe-leu-Pro-Ala-Ala) is further assembled using *in situ* neutralisation protocols. The N-terminus is deprotected with TFA and the C-terminal allyl protection group removed using $Pd[P(Ph)_3]_4$ as described. The cyclisation is then performed with BOP/DIEA in DMF and the product cleaved from the resin by photolysis.

Example 10 Assembly of a Difficult Peptide Sequence

We have previously reported that the standard Fmoc assembly of the peptide STAT91 (699-709) was troublesome (Alewood *et al*, 1997). In this experiment we employ the photolabile auxiliary to improve the Fmoc SPPS assembly of the linear peptide in the following way:



The peptide sequence LISV is assembled on WANG resin using standard Fmoc SPPS protocols. The auxiliary is introduced on the leucine residue as previously described. The rest of the target sequence is then assembled using the standard Fmoc protocols, and the product cleaved from the resin with TFA. The auxiliary-containing product is analysed by HPLC and MS, and the purity compared with our previously obtained results. The auxiliary is then removed by photolysis in DMF in the presence of excess Scavenger.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

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